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# *OXI1* kinase plays a key role in resistance of *Arabidopsis* towards aphids (*Myzus persicae*)

Tahsin Shoala · Martin G. Edwards · Marc R. Knight · Angharad M. R. Gatehouse

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**Abstract** Plants have co-evolved with a diverse array of pathogens and insect herbivores and so have evolved an extensive repertoire of constitutive and induced defence mechanisms activated through complex signalling pathways. *OXI1* kinase is required for activation of mitogen-activated protein kinases (MAPKs) and is an essential part of the signal transduction pathway linking oxidative burst signals to diverse downstream responses. Furthermore, changes in the levels of *OXI1* appear to be crucial for appropriate signalling. Callose deposition also plays a key role in defence. Here we demonstrate, for the first time, that *OXI1* plays an important role in defence against aphids. The *Arabidopsis* mutant, *oxi1-2*, showed significant resistance both in terms of

population build-up ( $p < 0.001$ ) and the rate of build-up ( $p < 0.001$ ). *Arabidopsis* mutants for  $\beta$ -1,3-glucanase, *gns2* and *gns3*, showed partial aphid resistance, significantly delaying developmental rate, taking two-fold longer to reach adulthood. Whilst  $\beta$ -1,3-glucanase genes *GNS1*, *GNS2*, *GNS3* and *GNS5* were not induced in *oxi1-2* in response to aphid feeding, *GNS2* was expressed to high levels in the corresponding WT (Col-0) in response to aphid feeding. Callose synthase *GSL5* was up-regulated in *oxi1-2* in response to aphids. The results suggest that resistance in *oxi1-2* mutants is through induction of callose deposition via MAPKs resulting in ROS induction as an early response. Furthermore, the results suggest that the  $\beta$ -1,3-glucanase genes, especially *GNS2*, play an important role in host plant susceptibility to aphids. Better understanding of signalling cascades underpinning tolerance to biotic stress will help inform future breeding programmes for enhancing crop resilience.

**Keywords** Aphid resistance/susceptibility · Oxidative burst · Oxidative signal inducible 1 (*OXI1*) kinases · Callose synthase (*GSL*) ·  $\beta$ -1,3-Glucanase (*GNS*) · *Arabidopsis* mutants · Differential gene expression

T. Shoala · M. G. Edwards · A. M. R. Gatehouse (✉)  
School of Natural and Environmental Sciences, Newcastle  
University, Newcastle-Upon-Tyne NE1 7RU, UK  
e-mail: a.m.r.gatehouse@ncl.ac.uk

T. Shoala  
e-mail: tahsen.shoala@must.edu.eg

M. G. Edwards  
e-mail: martin.edwards@newcastle.ac.uk

T. Shoala  
College of Biotechnology, Misr University for Science  
and Technology, Giza, Egypt

M. R. Knight  
School of Biological and Biomedical Sciences, Durham  
University, Durham DH1 3LE, UK  
e-mail: m.r.knight@durham.ac.uk

## Introduction

Aphids are economically important pests globally causing extensive crop damage both as a result of nutrient abstraction, but, more importantly as disease vectors. The plant–aphid interaction is complex and highly dynamic, subject to continual change (Mello and Silva-Filho 2002). Recently, studies have shown that aphids secrete effectors that modulate plant defence responses (Bos et al. 2010; Atamian et al. 2013; Pitino and Hogenhout 2013) pointing to parallels between plant–pathogen and plant–insect interactions (Jaouannet et al. 2014). It has been suggested that two different processes are involved in the elicitation of plant defence. One involves a gene-for-gene recognition of aphid-derived elicitors by plant resistance genes, followed by the activation of aphid resistance and defence responses. The other involves recognition of aphid-damaged tissue leading to changes in plant chemistry, followed by the production of signalling molecules that trigger a general stress response, similar to the basal plant defence to pathogens (Smith and Boyko 2007). The activation of signalling pathways in response to phloem-feeding aphids alters gene expression, which in turn leads to changes in the molecular composition within the cell. Transcriptional and proteomic analyses have indicated that encoded proteins of these differentially regulated genes function in direct defence, defence signalling, oxidative burst, secondary metabolism, cell maintenance and photosynthesis (Zhu-Salzman et al. 2004; Ferry et al. 2011; Guan et al. 2015). There is also evidence of crosstalk between different plant signalling pathways resulting in plants subjected to abiotic stress being more resistant to biotic stress. For instance, the phytohormone abscisic acid (ABA), an important regulator of plant responses to osmotic stress imposed by drought and salt, interacts in a complex, but well understood way with SA, JA and ET (Pineda et al. 2013). Cytokinins (CKs) play important roles in plant biotic interactions and in the regulation of complex source–sink relationships structuring plant-based food webs (Giron et al. 2013).

Active oxygen species (AOS) can function as signalling molecules leading to specific downstream events, playing key roles in plants in response to stress e.g. pathogen attack (Levine et al. 1994) and wounding (Orozco-Cárdenas et al. 2001). Oxidative signal inducible 1 (*OXI1*) is a serine/threonine kinase related

to the protein kinase family (AGC) (Alessi 2001; Bogre et al. 2003) required for the partial activation of mitogen-activating protein kinases 3 and 6 (MPK3 and MPK6) (Rentel et al. 2004) and has been shown to be an essential component of the signal transduction pathway linking oxidative burst signals to diverse downstream processes. This is supported by the findings that *Arabidopsis* null *oxi1* mutants were more susceptible than WT plants to the bacterial pathogen *Pseudomonas syringae* so confirming its role in plant immunity. The level of *OXI1* expression appears to be critical in mounting an appropriate defence response since these authors also showed that *OXI1* overexpressing lines also display increased susceptibility to biotrophic pathogens; the induction of *OXI1* following *P. syringae* infection was found to be correlated with the oxidative burst and that ROS produced through NADPH oxidases drives *OXI1* expression during the plant–pathogen interaction (Petersen et al. 2009). The endophytic fungus *Piriformospora indica* is known to promote plant growth and enhance resistance to certain plant pathogens and there is now evidence that this is mediated via the *OXI1* kinase pathway and that  $H_2O_2$  formation is reduced (Camehl et al. 2011).

Not only is *OXI1* responsive to pathogen attack but also to other external stimuli such as  $H_2O_2$  (Rentel et al. 2004). Interestingly, Pei et al. (2000) showed that ABA-induced  $H_2O_2$  production and the  $H_2O_2$ -activated  $Ca^{2+}$  channels are important mechanisms for ABA-induced stomatal closing thus enabling the plants to respond to drought. Thus stomatal opening and closure is controlled by the concentration of cytosolic  $Ca^{2+}$ . Cytosolic  $Ca^{2+}$  also stimulates the synthesis and deposition of callose ( $\beta$ -1,3-glucan), an important component of the plant defence system both in response to biotic and abiotic stress (Verma and Hong 2001), including both pathogen infection and insect attack (Aidemark et al. 2009) where its deposition at the sieve plates (McNairn and Currier 1967) regulates intercellular transport of molecules by controlling the size exclusion limit of plasmodesmata as a response to developmental signals or environmental signals, e.g. wounding and pathogen attack (Kauss 1996; Köhle et al. 1985). Not only does callose deposition strengthen the cell wall at the site of attack (Aist 1976; Bell 1981), but in resistant carnation cultivars it has been found in plasmodesmata in non-infected cells bordering the infected site so preventing the spread of fungal infection in resistant cultivars

(Trillas et al. 2000). Callose deposition can also be synthesized in response to ABA, and other physiological stresses (Stone and Clarke 1992). Recent studies have reported that callose synthase 7 (*CSL7* or *GSL7*) is responsible for callose deposition in the sieve plates and that Arabidopsis *gsl7* mutants were unable to produce callose in the sieve pores in response to stresses (Xie and Hong 2011; Barratt et al. 2011; Xie et al. 2011).

$\beta$ -1,3-Glucanases are a class of hydrolytic enzymes that hydrolyse the 1,3- $\beta$ -D-glucosidic linkages in  $\beta$ -1,3-glucans (callose) and are recognised as one of the commonly known pathogenesis-related (PR) proteins, belonging to the PR2 group; they are rapidly induced by pathogenic infections but also by salicylic acid (SA), Jasmonic acid (JA) and ethylene (ET). Not only are  $\beta$ -1,3-glucanases induced by pathogen infection, but they exhibit antifungal activity, hydrolysing the fungal  $\beta$ -1,3-glucans, a major cell wall structural component of both fungi and plants (Leubner-Metzger and Meins 1999; Van Loon et al. 2006). In Arabidopsis the expression of PR1 and  $\beta$ -1,3-glucanase 2 (*GNS2*) genes increases in response to aphid (*Myzus persicae*) feeding; furthermore, following exposure to the signalling molecule salicylic acid (SA), transcription of *PR1* and *GNS2* was increased 10- and 23-fold respectively (Moran and Thompson 2001).

The present study demonstrates the involvement of *OXII*, *GNS1*, *GNS2* and *GNS3* in the interaction between a model plant-aphid system at both the phenotypic and molecular levels. Aphid survival is reported using a number of Arabidopsis mutants (*oxil-2*, *oxil-1*, *gns1*, *gns2*, and *gns3*) in comparison to their wild type backgrounds and correlated with transcript levels for selected callose synthase and  $\beta$ -1,3-glucanase genes in these mutant lines. The results suggest that resistance in *oxil* mutants is through induction of callose deposition via MAPKs resulting in ROS induction as an early response. Furthermore, that  $\beta$ -1, 3-glucanase genes, especially *GNS2*, play an important role in host plant susceptibility to aphids.

## Materials and methods

### Plant materials and growth conditions

**Arabidopsis thaliana mutants** Two different ecotypes were used, Columbia (Col-0) and Wassilewskija (Ws).

Two mutant lines for the *OXII* gene were investigated; *oxil-1*, background Ws, is a null mutant with an insertion in the beginning of the coding region, from the Salk collection, whilst *oxil-2* (in Col-0), from the Wisconsin collection, has an insertion in the promoter and exhibits severely reduced function (about 5% transcript level compared to WT) (Rentel et al. 2004; Petersen et al. 2009). Arabidopsis  $\beta$ -1,3-glucanase mutants (*gns1*, *gns2* and *gns3* (Salk) in Col-0; stock numbers N633409; N587824; N609800; N60000, respectively) were obtained from the Arabidopsis Biological Resource Centre (Nottingham Arabidopsis Stock Centre). Plants were grown in John Innes compost number 2 in controlled environment chambers under a 16:8 h light/dark cycles at 23 °C, 55% relative humidity.

### Aphid bioassays

Prior to the start of the bioassays, aphids (peach-potato aphid *Myzus persicae*) were reared either on Arabidopsis Col-0 or Ws. All bioassays were carried out on plants that were between 25 to 30 days old (i.e. rosette stage 5–10 leaves) maintained under growing conditions described above. Aphid performance was investigated by measuring the intrinsic rate ( $R_m$ ) of population build-up on the following Arabidopsis mutants together with their respective WT as controls: *oxil-1*, *oxil-2*, *gns1*, *gns2*, *gns3*. Two adult females were placed on each plant (at 5–10 leaf stage; all plants were isolated from each other) and allowed to produce nymphs. On the onset of nymph production, with the exception of two nymphs, all aphids including the original adults were removed and population build-up from the two remaining nymphs was monitored every 2 days for the duration of the study; zero time point (day 1) started when the two nymphs became adults. All bioassays were conducted concurrently and 15 plants were used for each line;  $n = 15$ ).

### Gene expression studies in Arabidopsis mutants in response to aphid feeding over time

For gene expression studies all Arabidopsis plants, using the same lines as described for the aphid bioassays above, were between 25 to 30 days old (i.e. rosette stage 5–10 leaves). Each plant was infested with 10 aphids for a range of different times

(3, 6, 12, 24 and 48 h) after which the aphids were removed and leaves immediately harvested and flash frozen; three time points (0, 3 and 48 h) for non-infested plants were used as controls. Relative expression was measured for: (1) callose synthases genes *GSL1*, *GSL5* both in *oxil-1*, *oxil-2* and their respective WT, Ws and Col-0; and (2)  $\beta$ -1,3-glucanase gene *GNS2* in *oxil-1*, *oxil-2* and their respective WT, Ws and Col-0; three Arabidopsis plants were used per line giving three individual biological replicates/line/gene.

#### Reverse transcription quantitative PCR (RT-qPCR)

Total RNA was isolated from ca. 200 mg of frozen leaf tissue using Trizol Reagent (Invitrogen), according to the manufacturer's protocols. The concentration and purity of the RNA samples was determined using a Nanodrop (ND-1000 Spectrophotometer; Nanodrop Technologies). All samples had an absorbance ratio (absorbance at A260/A280 nm) of between 1.9 and 2.2. Following quantification, all RNA samples were normalized to 100 ng  $\mu\text{l}^{-1}$ . To ensure maximum specificity and efficiency during qPCR amplification under a standard set of reaction conditions, Allele ID 7.7 software was used to design RT-qPCR primers (Table 1). One-step brilliant II sybr green master mix was used for RT-qPCR. Gradient PCR was used to identify the annealing temperature. A 25- $\mu\text{l}$  reaction volume PCR was employed using 1  $\mu\text{l}$  RNA at 100 ng  $\mu\text{l}^{-1}$ , 12.5  $\mu\text{l}$  of one step Master Mix (Agilent), 2 pmol each gene-specific primer, and UltraPure DNase/RNase-free distilled water (Qiagen) to 25  $\mu\text{l}$ . Amplification of RNA employed the following conditions: 30 min incubation at (50–60 °C) to allow the reverse transcription, initial 10 min of denaturation at 94 °C; followed by 39 cycles of denaturation 94 °C for 30 s, annealing at (50–60 °C) for 30 s, and extension at 72 °C for 45 s; followed by a final extension for 5 min at 72 °C. For normalisation the elongation factor (EF) gene was used as an endogenous control as it gave consistent expression levels. Three independent biological repeats and three independent technical repeats were performed for each gene/time point and threshold cycle reactions determined using Opticon Monitor software. Relative expression was calculated according to Pfaffl (2001).

**Table 1** Specific designed primers for QRT-PCR

Gene	Accession number	Primer sequences (5'–3') forward	Primer sequences (5'–3') reverse	Amplicon
<i>EF-1<math>\alpha</math></i>	AT5G60390	TGAGCAGCTCTCTTGCTTTCA	GGTGGTGGCATCCATCTTGTTACA	147 bp
<i>UBQ10</i>	AT4G05320	GGCCTTGATATAATCCCTGATGAATAAG	AAAGAGATAACAGGAACGGAACATAGT	120 bp
<i>TIP41</i>	AT4G34270	GTGAAAACCTGTTGGAGAGAAAGCAA	TCAACTGGATACCCCTTTCGCA	127 bp
<i>HELICASE</i>	AT1G58050	CCATTCTACTTTTTGGCGGCT	TCAATGGTAACTGATCCACTCTGATG	140 bp
<i>AtGNS1</i>	AT3G57270	GAGATGTTATGGTGGTAATGGA	GCTGAAGTAAAGTGTAGAGGTT	89 bp
<i>AtGNS2</i>	AT3G57260	ACCAATGTTGATGATTCCTCTC	CCGTAGCATACTCCGATT	81 bp
<i>AtGNS3</i>	AT3G57240	GATAATGCGAGAACTTAT	ATACTTAGGCTGTAGATT	177 bp
<i>AtGNS5</i>	AT5G20340	ACAACAATAGTGACTTCGTAA	AGGAGACCGTAGTTCAAG	85 bp
<i>AtGSL1</i>	AT1G05570	ATTGATGAACATATTGAGAAGGA	GATTAGCCGGAACGAACCTG	90 bp
<i>AtGSL5</i>	AT2G13680	TCTGTTGCTTGTTCCTTAT	CCAATGCTATCGGTATCTT	92 bp

## Statistical analysis

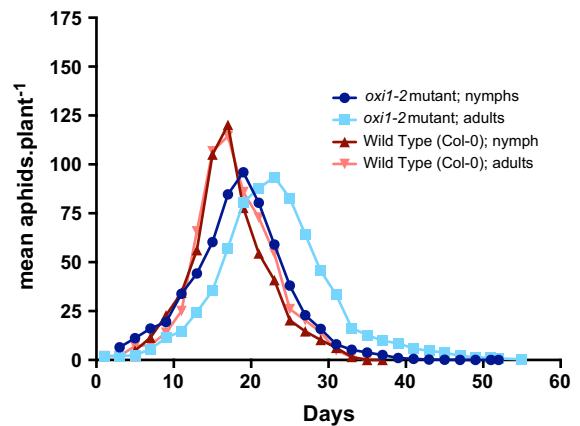
Repeated measures ANOVA were conducted for aphid bioassays. Two-way ANOVA with replication was used to test the  $p$  value of qRT-PCR results followed by Tukey test.  $*p < 0.05$ ,  $**p < 0.01$  and  $***p < 0.001$ .

## Results

Arabidopsis mutants show enhanced resistance to aphids

Aphid population build-up was measured on two Arabidopsis mutant lines and compared to wild type control plants (Col-0). On control plants nymph production was seen to increase over the course of the bioassay reaching a population maximum of 120 at day 17, thereafter nymph production declined. In contrast, on *oxil-2*, nymph production was significantly lower although the duration was longer ( $p < 0.001$ ). Moreover there was a shift in the reproductive peak, this being delayed by 4 days on the mutant line (Fig. 1). In comparison, the number of adults on Col-0 peaked at day 19 with 114 adult aphids, with no survivors after 37 days. However, on *oxil-2*, the number of adult aphids was significantly lower after 19 days with 80 adults ( $p < 0.001$ ). Interestingly, as with nymph production there was a shift in the developmental peak to adulthood by 4 days. (Fig. 1). The population dynamics of the adults thus followed those exhibited by the nymphs demonstrating that the *oxil-2* mutant exhibited enhanced resistance to aphid infestation.

Aphid performance on the wild type was also compared to that on the *gns* mutant lines. Whilst there were no significant differences in terms of the number of nymphs produced on *gns1*, there was a shift in the reproduction peak by 4 days, as seen with *oxil-2*, with no aphids in the nymphal stages after 47 days (Fig. 2a). Again, as seen on *oxil-2*, the mean number of adults was significantly lower on *gns1* after 19 days with 84 adult aphids ( $p < 0.001$ ). However, in contrast to either the WT or *oxil-2* the maximum population density (116 adults) was not reached for a further 10 days, thus demonstrating a very large shift in the developmental peak; thereafter the number of adults rapidly decreased (Fig. 2a). For *gns2* (Fig. 2b) and *gns3* (Fig. 2c) the delay in the reproductive peaks were even greater, with



**Fig. 1** Arabidopsis mutant *oxil-2*, shows enhanced resistance to aphids (*Myzus persicae*). Population build-up of aphids on the *oxil-2* mutant and corresponding wild type plants (Col-0). Bioassays measured the intrinsic rate of increase ( $R_m$ ) of the population. Two adult females were placed on each plant (at 5–10 leaf stage) and allowed to produce nymphs. On the onset of nymph production, with the exception of two nymphs, all aphids including the original adults were removed and population build-up was monitored every 2 days. The data represents the mean number of aphids per plant. Values are means of 15 plants  $\pm$  SE ( $n = 15$ ). Data was analysed by two-way ANOVA. Significant differences in the number of adults are indicated by  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$

an approximate doubling of the time taken, compared to their WT controls. However, the numbers of nymphs produced were greater, particularly for *gns3*.

Relative expression of callose synthase genes *GSL1* and *GSL5* in *oxil-2* and the WT (Col-0) in response to aphid feeding

Expression levels for *GSL1* in non-infested WT and *oxil-2* plants remained low over the duration of the assay (48 h), with relative expression levels being 1.2-fold and 2.5-fold, respectively, at both 3 h and 48 h compared, to 0 h. However, expression was significantly enhanced in both lines in response to aphid feeding and at 3 h post feeding was significantly greater in *oxil-2* compared to WT, being 14.6-fold and 8.4-fold greater than in the absence of aphid feeding (Fig. 3a). Irrespective of the line, maximal expression occurred 6 h post feeding, but at this stage was significantly ( $p < 0.01$ ) greater in the control compared to the mutant line, with relative expression being 27.2-fold and 14.3-fold, respectively. After 24 h, relative expression levels declined rapidly.

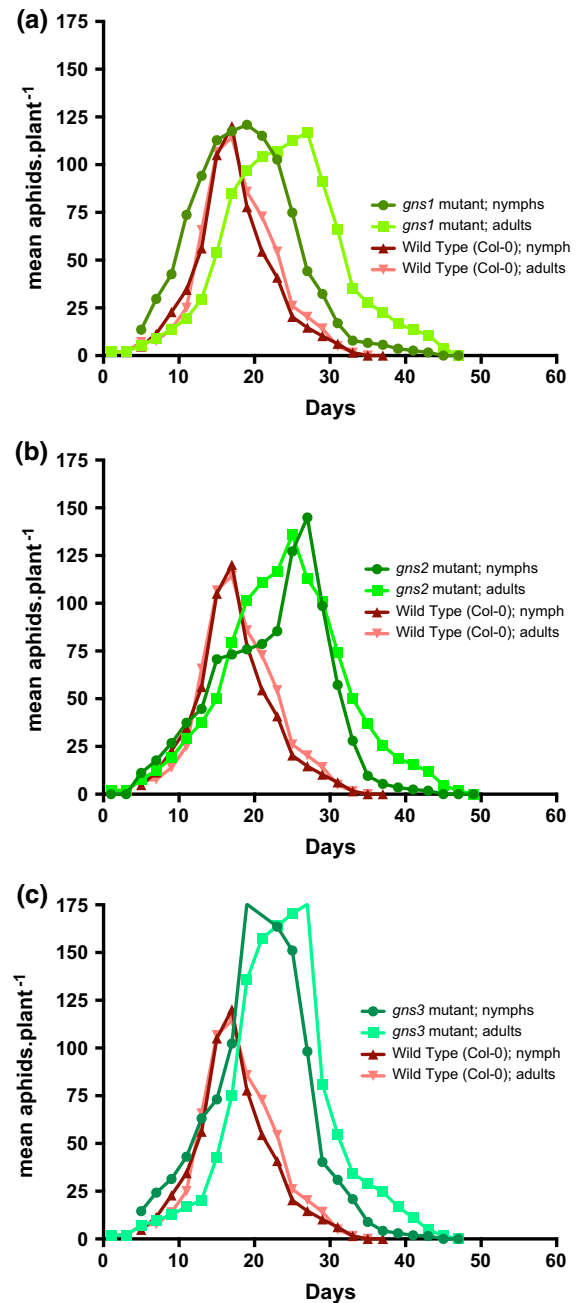


**Fig. 2** The Arabidopsis mutants *gns1*, *gns2* and *gns3* have a significant effect on aphid (*Myzus persicae*) performance, delaying the reproductive and developmental peaks. Population build-up of aphids on **a** the *gns1* mutant; **b** the *gns2* mutant and **c** *gns3* mutant compared to their corresponding WT plants (Col-0). Bioassays measured the intrinsic rate of increase ( $R_m$ ) of the population. Two adult females were placed on each plant (at 5–10 leaf stage) and allowed to produce nymphs. On the onset of nymph production, with the exception of two nymphs, all aphids including the original adults were removed and population build-up was monitored every 2 days. The data represents the mean number of aphids per plant. Values are means of 15 plants  $\pm$  SE ( $n = 15$ ). Data was analysed by two-way ANOVA. Significant differences in the number of adults are indicated by \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$

Expression levels for *GSL5* were far more consistent than for *GSL1*, and although expression for this gene were increased in both the WT and mutant line in response to aphid feeding, expression was always significantly greater in *oxil-2* compared to WT, with maximal expression 12 h post infestation where relative expression levels were 6.6-fold and 1.8-fold respectively (Fig. 3b). As for *GSL1*, expression levels of *GSL5* in non-infested WT and *oxil-2* mutant plants remained low over the duration of the assay (48 h).

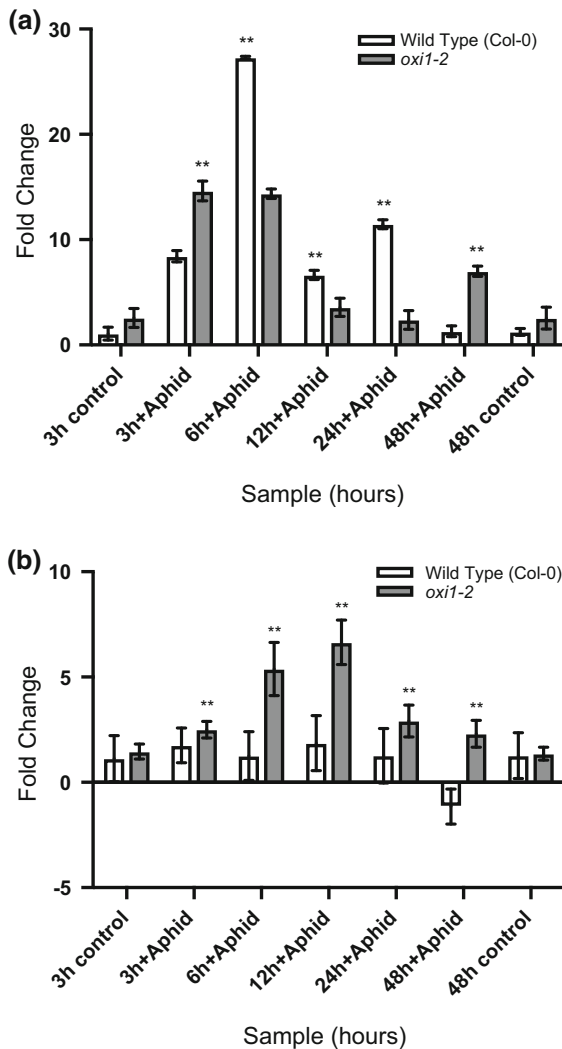
Relative expression of callose synthase genes *GSL1* and *GSL5* in *oxil-1* and the WT (Ws) in response to aphid feeding

Background levels for both *GSL1* and *GSL5* expression in *oxil-1* and its WT in the absence of aphid feeding remained low and moderately constant throughout the 48 h time period of the trial (Figs. 3b, 4a). As with expression of *GSL1* in the *oxil-2* mutant, expression of this gene in *oxil-1*, representing a different genetic background, resulted in increased expression in response to aphid feeding, with maximal expression occurring 12 h post feeding, and relative expression levels being 11.2-fold higher; however, in contrast to *oxil-2*, expression levels for this particular gene remained high, even after 48 h (7.1-fold higher). Surprisingly, the relative expression of *GSL1* in the WT Ws behaved differently to WT Col-0 in that aphid feeding overall caused down regulation of this gene (Fig. 4a). In both the Ws and the null mutant *oxil-1*, aphid feeding caused highly significant ( $p < 0.001$ ) down regulation of expression of *GSL5* over the 48 h time period, and was greatest in *oxil-1* 48 h post infestation (Fig. 4b).



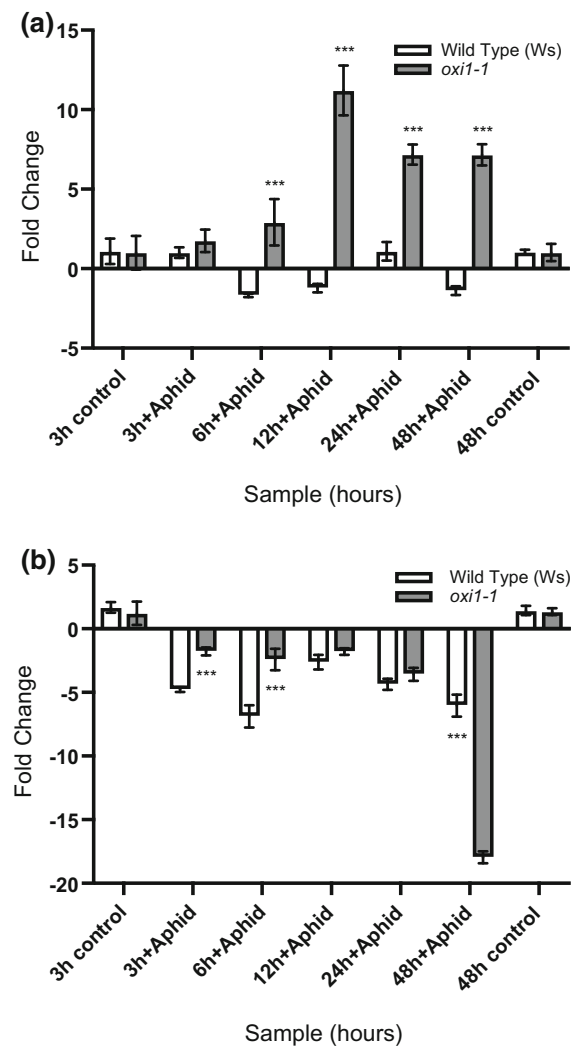
Relative expression of  $\beta$ -1,3-glucanase genes *GNS1*, *GNS2*, *GNS3*, and *GNS5* in *oxil-2* and its WT (Col-0) in response to aphid feeding

$\beta$ -1,3-Glucanase genes (*GNS1*, *GNS2*, *GNS3* and *GNS5*) did not appear to be expressed in *oxil-2*, irrespective of whether the plants had been infested with aphids or not (Fig. 5a). Furthermore, in the WT



**Fig. 3** Aphid (*Myzus persicae*) infestation has a significant effect on expression levels of callose synthase genes (*GSL1*, *GSL5*) in Arabidopsis cv Columbia, enhancing expression of *GSL5* in *oxi1-2* relative to the WT. Relative expression of: **a** *GSL1* and **b** *GSL5* in *oxi1-2* compared to the wild type (Col-0), in response to aphid feeding. Total RNA was extracted from Arabidopsis leaves (ca. 200 mg fresh wt from rosette stage 5–10 leaves) at a series of time points (3, 6, 12, 24 and 48 h) post infestation and from non-infested plants (at time points 0, 3 and 48 h). Expression levels of target genes for both treatments were compared to those of non-infested plants at 0 h; the Arabidopsis elongation factor (*EF*) gene was used as the reference gene. Data was analysed by two-way ANOVA. Significant differences in gene expression are indicated by \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . Data are mean  $\pm$  SE (n = 9)

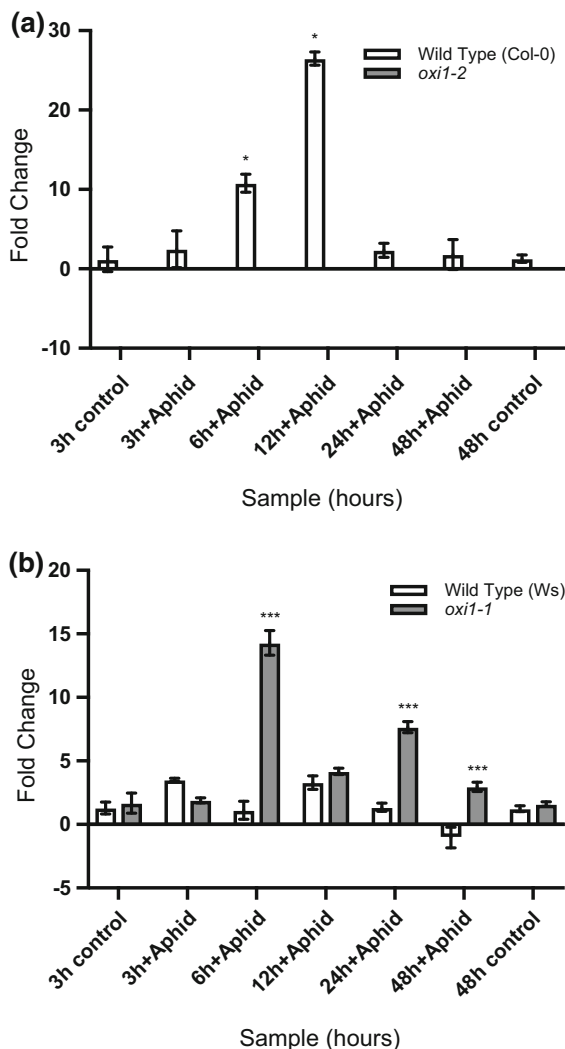
(Col-0), *GNS1*, *GNS3* and *GNS5* were expressed at such low levels making it difficult to quantify gene expression accurately and reliably. However,



**Fig. 4** Aphid (*Myzus persicae*) infestation has a significant effect on expression levels of callose synthase genes (*GSL1*, *GSL5*) in Arabidopsis cv Wassilewskija, enhancing expression of *GSL1* in the *oxi1-1* relative to the WT, (Ws), but significantly suppressing expression of *GSL5*. Relative expression of: **a** *GSL1* and **b** *GSL5* in *oxi1-1* compared to the corresponding wild type, in response to aphid feeding. Total RNA was extracted from Arabidopsis leaves (ca. 200 mg fresh wt from rosette stage 5–10 leaves) at a series of time points (3, 6, 12, 24 and 48 h) post infestation and from non-infested plants (at time points 0, 3 and 48 h). Expression levels of target genes for both treatments were compared to those of non-infested plants at 0 h; the Arabidopsis elongation factor (*EF*) gene was used as the reference gene. Data was analysed by two-way ANOVA. Significant differences in gene expression are indicated by \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . Data are mean  $\pm$  SE (n = 9)

expression of *GNS2* was readily detected in Col-0, and whilst the expression levels remained low and constant throughout the 48 h time course in non-





**Fig. 5** The effect of aphid (*Myzus persicae*) infestation on relative expression levels of the  $\beta$ -1,3-glucanase 2 gene (*GNS2*) in the *oxil* mutants is influenced by the genetic background. Relative expression of *GNS2* in: **a** *oxil-2* compared to the corresponding WT (Col-0) and **b** *oxil-1* compared to its corresponding WT (Ws) in response to aphid feeding over time. Total RNA was extracted from Arabidopsis leaves (ca. 200 mg fresh wt from rosette stage 5–10 leaves) at a series of time points (3, 6, 12, 24 and 48 h) post infestation and from non-infested plants (at time points 0, 3 and 48 h). Expression levels of target genes for both treatments were compared to those of non-infested plants at 0 h; the Arabidopsis elongation factor (*EF*) gene was used as the reference gene. Data was analysed by two-way ANOVA. Significant differences in gene expression are indicated by \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . Data are mean  $\pm$  SE ( $n = 9$ )

infested plants, relative expression significantly increased at 6 h post feeding, with maximal

expression at 12 h (26.4-fold;  $p < 0.001$ ), before rapidly falling back to basal levels (Fig. 5a).

Relative expression of  $\beta$ -1,3-glucanase genes *GNS1*, *GNS2*, *GNS3* and *GNS5* in *oxil-1* and its WT (Ws) in response to aphid feeding

As with *oxil-2* and its WT (Col-0), expression of genes encoding *GNS1*, *GNS3* and *GNS5* genes were not detected in *oxil-1* in response to aphid feeding, neither were they detected in the WT. In contrast, *GNS2* was detected at low levels in both *oxil-1* and the WT (Ws), remaining low throughout the 48 h time course in the absence of aphid feeding. Whilst *GNS2* expression remained low in the WT plants in response to aphid feeding, expression was significantly enhanced in the null mutant, with maximal expression (14.2-fold) occurring 6 h post feeding, thereafter showing a decline with relative expression levels of 2.9-fold at 48 h (Fig. 5b). Interestingly, at this same time point, expression was down-regulated in the corresponding WT, in response to aphid feeding (Fig. 5b). These results suggest that for  $\beta$ -1,3-glucanase *GNS2*, the genetic background may have an effect on subsequent gene expression in that it is not expressed in *oxil-2* in response to aphid feeding but is in *oxil-1*.

## Discussion

The potential roles of oxidative signal inducible 1 (OXI1) kinases,  $\beta$ -1,3-glucanase (*GNS*) and callose synthase (*GSL*), and their interaction in enhancing resistance to aphids, was investigated in the present study using a range of Arabidopsis mutants. Bioassay results showed that *oxil-2* significantly ( $p < 0.001$ ) reduced the numbers of aphids produced, delaying both the developmental period and reproductive peak, thus reducing population build-up compared to the corresponding wild type (Col-0; Fig. 1a). Similar effects were also observed for the three different mutants of  $\beta$ -1,3-glucanase (Fig. 2) in that there was a delay in the rate of development, particularly for *gns2* and *gns3*, accompanied by a significant shift in the reproductive peak but, in contrast to *oxil-2*, aphid numbers were either the same as for their respective wild types (Col-0; *gns1*), or significantly ( $p < 0.001$ ) greater (*gns2*, *gns3*). Notably, despite the *gns* mutants

being able to support similar, or greater aphid densities compared to their wild types, the plants were able to tolerate this, remaining healthier and surviving much longer than the control plants (Fig. 2b, c), presumably as a result of these genes not being expressed in the mutant in response to aphid feeding.

Callose synthase is a key enzyme involved in the synthesis of  $\beta$ -1,3-glucan (callose), whilst  $\beta$ -1,3-glucanase is involved in the breakdown of this compound. Their roles in plant defence are not surprising and have been well documented, particularly in pathogen defence. For example, *GSL5*, which is required for wound and papillary callose formation (Jacobs et al. 2003) at sites of pathogen attack, has been shown to increase resistance of Arabidopsis to powdery mildew (Jacobs et al. 2003; Nishimura et al. 2003; Kim et al. 2005). Studies using pathogen elicitors have resulted in similar effects. Treatment of Arabidopsis leaves with chitosan, an elicitor allied with fungal pathogens, showed callose deposition in *gsl5* disruption mutants (El Hadrami et al. 2010), whilst application of flagellin (*flg22*), derived from a bacterial pathogen, resulted in *GSL5* callose deposition (Gomez-Gomez et al. 1999; Luna et al. 2011). More recent studies using *GSL5*-overexpressing Arabidopsis lines demonstrated that callose synthase activity was four-fold higher than in wild-type plants 6 h post inoculation with the virulent powdery mildew *Golovinomyces cichoracearum*. This increase in activity was correlated with enlarged callose deposits at the sites of attempted fungal penetration (Ellinger et al. 2013). Furthermore, haustoria formation was prevented in these resistant transgenic lines during infection, and neither the salicylic acid-dependent nor jasmonate-dependent pathways were induced. Subsequently this group also reported the exposition of a callose layer on the surface of the pre-existing cellulosic cell wall facing the invading pathogen and suggested that the importance of this previously unknown polymeric defence network was to prevent cell wall hydrolysis and penetration by the fungus (Eggert et al. 2014). Will and Van Bel (2006) have also reported the involvement of callose synthase in response to pathogen attack and wounding. In the present study the *oxil-2* mutant was shown to be significantly more resistant to aphid infestation compared to wild type plants. Interestingly, expression of *GSL5* in these plants was significantly up-regulated in response to aphid feeding, with maximal

expression 24 h post infestation (Fig. 3b). This would suggest that callose synthase is playing a major role in the observed resistance and is in agreement with pathogen studies where this same gene, *GSL5*, has been over-expressed. These findings are also in agreement with Lu et al. (2011) who reported the role of glucan synthase in callose deposition and aphid resistance, again implicating the role of *GSL5* in host-plant resistance. Whilst a direct link between expression of *GSL5* and callose deposition was not investigated in the present study, numerous other studies have demonstrated a direct correlation between the expression of this gene and callose accumulation.

OXI1 is a serine/threonine kinase induced in response to a wide range of Reactive Oxygen Species (ROS), especially hydrogen peroxide (Rentel et al. 2004), and is required for full activation of MAPKs genes, especially MAPK3 and MAPK6. These genes are involved in plant defence against bacterial and fungal pathogens. Full activation of MAPK cascades trigger the calcium pathway (Asai et al. 2002), which in turn stimulates callose synthase activity followed by callose production, leading to enhanced resistance to biotic stress. OXI1 plays an essential role in the signal transduction pathway connecting oxidative burst signals to different downstream responses (Petersen et al. 2009); it thus facilitates signal transduction of ROS (Kwak et al. 2003). The *oxil* mutant has unique features including continuous induction of MAPK kinase genes, early response ROS accumulation and signal transduction, and increased callose synthase and catalase activity (Petersen et al. 2009). These features not only enable *oxil-2* to tolerate moderately high aphid populations, but, importantly confer greater resistance than the wild types by decreasing aphid numbers, and delaying the rate of development so shifting the reproductive peak, as seen in the present study. In contrast the wild type (Col-0), which was susceptible to aphid infestation supporting a rapid population increase in the short term, was unable to survive such high levels of aphid infestation and died. These wild type plants showed significant upregulation of  $\beta$ -1,3-glucanase gene *GNS2* in response to aphid feeding (Fig. 5a), but low expression of *GSL5* (Fig. 3b) post aphid feeding, again consistent with their proposed roles in plant defence. However, the *oxil-1* null mutant (Ws) showed significant up-regulation of  $\beta$ -1,3-glucanase *GNS2* and down-regulation of callose synthase *GSL5* which may result in plant

susceptibility in response to aphid feeding. Previous studies have shown that this *oxi1-1* (Ws) null mutant exhibits enhanced susceptibility when challenged with virulent *Hyaloperonospora parasitica* (Rentel et al. 2004). These findings are consistent with the hypothesis that callose synthase is involved in enhancing plant defence through production of callose deposits whilst  $\beta$ -1,3-glucanases, responsible for callose degradation, are involved in susceptibility since they enable sap-sucking insect such as aphids, to abstract nutrients from the phloem cells. Furthermore, Hao et al. (2008) demonstrated that the brown planthopper *Nilaparvata lugens* is able to unplug callose deposition in the sieve pores by activating  $\beta$ -1,3-glucanases in rice plants. Whether aphids also stimulate filament formation of sieve element occlusion (SEO) proteins (Batailler et al. 2012) or cause callose degradation, is currently unknown.

In Arabidopsis, *PR1* and  $\beta$ -1,3-glucanase *GNS2*, both associated with the salicylic acid dependent response to pathogens, have been shown to be induced in response to aphid (*M. persicae*) feeding with increased transcript levels of 10 and 23 fold, respectively (Moran and Thompson 2001). Arabidopsis plants challenged with both virulent and avirulent strains of the pathogen *Pseudomonas syringae* pv. *tomato* DC3000 induced the expression of *OXII*; furthermore, *PR1* has been induced in response to a diverse range of *P. syringae* isolates and linked to the observed resistance to this bacterial pathogen (Dempsey et al. 1999). Interestingly, Arabidopsis ascorbate-deficient mutants showed microlesions, constitutive *PR* gene expression in response to *P. syringae* infection, and although these mutants were deficient in induction of  $H_2O_2$  sensitive genes, they exhibited increased pathogen resistance (Pavet et al. 2005). Results from the present study suggest that  $\beta$ -1,3-glucanases are involved in host plant susceptibility to aphids since the Arabidopsis mutants (*gns1*, *gns2* and *gns3*) all showed enhanced tolerance in terms of delaying aphid development but also being able to withstand greater aphid densities, surviving much longer than the control plants. These findings are in keeping with Anderson et al. (2014) who suggest a role for (1,3;1,4)- $\beta$ -glucanase in susceptibility of wheat to Russian wheat aphid. This hypothesis could be tested using RNAi.

The role of *OXII* in plant defence is clearly very complex. For example, Petersen et al. (2009) have

shown that both reduced expression and overexpression led to enhanced susceptibility to biotrophic pathogens using the same mutants as in the present study. Since it is unlikely that a single kinase plays both a negative and positive role during the same defence, these authors proposed that it was the actual level of *OXII* protein that was crucial for the appropriate signalling, and that modulation of these levels (either higher or lower) disrupts *OXII* function.

The present study demonstrates that resistance in *oxi1* mutants is through MAPK induction of callose deposition resulting in ROS induction as an early response. In addition, it demonstrates the involvement of *OXII*, *GNS1*, *GNS2* and *GNS3* in the interaction between a model plant-aphid system at both the phenotypic and molecular levels. Furthermore, it demonstrates that  $\beta$ -1,3-glucanase genes, especially *GNS2*, play an important role in host plant susceptibility to aphids. The data presented have important implications for future crop breeding programmes in terms of increasing host plant resistance, particularly with respect to enhanced resistance to sap-sucking insect pests.

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**Authors' contribution** TS carried out experimental work, data analysis, initial draft. MGE experimental design, data analysis, final draft, MRK intellectual input, strategic direction. AMRG overall concept, intellectual input, final draft.

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